Yolk proteolysis and aquaporin-1o play essential roles to regulate fish oocyte hydration during meiosis resumption

Mercedes Fabra a,b,c, Demetrio Raldúa b,c, María G. Bozzo c,d, Peter M.T. Deen e, Esther Lubzens f, Joan Cerdà a,b,c,*

a Lab IRTA-ICM, CMIMA (CSIC), Room B46, CMIMA-CSIC, Passeig Marítim 37-49, 08003-Barcelona, Spain
b Center of Aquaculture-IRTA, Tarragona 43540, Spain
c Reference Center in Aquaculture, Generalitat de Catalunya, Barcelona, Spain
d Department of Cell Biology, University of Barcelona, Barcelona 08071, Spain
e Department of Physiology, Radboud University Nijmegen Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands
f Israel Oceanographic and Limnological Research, National Institute of Oceanography, Haifa 31080, Israel

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Abstract

In marine fish, meiosis resumption is associated with a remarkable hydration of the oocyte, which contributes to the survival and dispersal of eggs and early embryos in the ocean. The accumulation of ions and the increase in free amino acids generated from the cleavage of yolk proteins (YPs) provide the osmotic mechanism for water influx into the oocyte, in which is involved the recently identified, fish specific aquaporin-1o (AQP1o). However, the timing when these processes occur during oocyte maturation, and the regulatory pathways involved, remain unknown. Here, we show that gilthead sea bream AQP1o (SaAQP1o) is synthesized at early vitellogenesis and transported towards the oocyte cortex throughout oocyte growth. During oocyte maturation, shortly after germinal vesicle breakdown and before complete hydrolysis of YPs and maximum K+ accumulation is reached, SaAQP1o is further translocated into the oocyte plasma membrane. Inhibitors of yolk proteolysis and SaAQP1o water permeability reduce sea bream oocyte hydration that normally accompanies meiotic maturation in vitro by 80% and 20%, respectively. Thus, yolk hydrolysis appears to play a major role to create the osmotic driving force, while SaAQP1o possibly facilitates water influx into the oocyte. These results provide further evidence for the role of AQP1o mediating water uptake into fish oocytes, and support a novel model of fish oocyte hydration, whereby the accumulation of osmotic effectors and AQP1o intracellular trafficking are two highly regulated mechanisms.

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Introduction

The oocytes of lower vertebrates are arrested at prophase of the first meiotic division during their long period of growth within the ovary, which is regulated by the circulating gonadotropin follicle-stimulating hormone (FSH) (Masui, 1985). When the growth phase is terminated, a surge of the gonadotropin luteinizing hormone (LH) triggers the synthesis and secretion of a maturation-inducing steroid (MIS) from the granulosa cells surrounding the oocyte, which in turn activates the maturation promoting factor (MPF) in the oocyte cytoplasm to resume meiosis (reviewed by Masui and Clark, 1979; Nagahama et al., 1995). In many teleosts, however, meiosis resumption (or oocyte maturation) is accompanied by important morphological changes, such as germinal vesicle breakdown (GVBD), lipid coalescence and clearing of the ooplasm (Wallace and Selman, 1981). In marine teleosts that produce buoyant (pelagic) eggs in seawater, termed pelagophils, the oocyte also undergoes a significant increase in size due to a rapid water uptake during the maturation process (Fulton, 1898; Wallace and Selman, 1981; Watanabe and Kuo, 1986; Craik and
Harvey, 1987; Mellinger, 1994; Thorsen and Fyhn, 1996; Selman et al., 2001; Finn et al., 2002a; Fabra et al., 2005). This unique physiological process among lower vertebrates contributes to the positive buoyancy of fish eggs and early embryos in the ocean, which is essential for their survival and dispersal.

During the growth period, the fish oocyte incorporates the circulating yolk precursors vitellogenins (Vgs), Vg1 and Vg2, which are processed into yolk proteins (YPs), such as lipovitellin (Lv), phosvitins and β-component (Wallace, 1985; Tyler et al., 1988; Matsubara and Koya, 1997; Matsubara et al., 1999; Hiramatsu et al., 2002a,b; Sawaguchi et al., 2005; LaFleur et al., 2005). In pelagophil teleosts, the YPs are stored in membrane-bound yolk globules that usually maintain their integrity throughout oocyte growth, and initially accrete within crystalline inclusions (Flegel, 1977; Lange, 1980; Kjesbu and Kryvi, 1989; Selman and Wallace, 1989; Wallace and Selman, 1990; Selman et al., 2001). During oocyte maturation, yolk globules fuse into a single mass of fluid yolk, concomitant with a second proteolytic event of Vg-derived YPs. This process increases the concentration of free amino acids (FAAs) and small peptides in the oocyte, thus providing an osmotic mechanism for water influx into the oocyte (Thorsen et al., 1996; Matsubara and Koya, 1997; Matsubara et al., 1999; Reith et al., 2001; Selman et al., 2001). Yolk hydrolysis, however, is not the only mechanism underlying oocyte hydration, since the accumulation of ions, such as K+ and Cl−, during oocyte maturation may also contribute to water uptake (Milroy, 1898; Craik and Harvey, 1984, 1986, 1987; Watanabe and Kuo, 1986; Thorsen and Fyhn, 1991; LaFleur and Thomas, 1991; Wallace et al., 1992; Selman et al., 2001; Finn et al., 2002a).

Despite that yolk cleavage and ion uptake seem to be the main osmotic effectors for oocyte hydration, the timing when these processes occur during maturation is not well established. In addition, the cellular mechanisms by which water could be rapidly transported into the oocyte have remained unknown. Aquaporins (AQPs) are constitutively open molecular channels that transport water and other solutes along an osmotic gradient, where the direction of water flow is determined by the orientation of the gradient (reviewed by Agre et al., 2002). In a recent study on the pelagophil gilt-head sea bream (Sparus aurata), a novel water-selective AQP highly expressed in the ovary, related to mammalian AQP1, was identified and named SaAQP1o (Fabra et al., 2005). The investigations into the physiological role of SaAQP1o have provided immunological and functional evidence suggesting that this water channel is localized in the oocyte and mediates water uptake during hydration (Fabra et al., 2005). Interestingly, functional water channels related to SaAQP1o, and also predominantly expressed in the ovary, have been discovered in other pelagophil teleosts (unpublished data). However, although the discovery of AQP1o has uncovered one of the key molecular events during fish oocyte hydration, the mechanisms by which these channels may be regulated during oocyte hydration remain unknown.

In order to explore potential regulatory pathways of SaAQP1o in the oocyte, in this study we investigated the pattern of SaAQP1o gene expression and subcellular localization during sea bream oocyte growth, as well as during MIS-induced meiosis resumption in vitro. The temporal changes in SaAQP1o localization during oocyte maturation, in relation to the events of GVBD, yolk processing, accumulation of inorganic ions, and oocyte swelling, were examined in detail. Finally, by using an H+-ATPase inhibitor to block the cleavage of YPs, and a non-toxic inorganic inhibitor of SaAQP1o-mediated water transport, the contributions of yolk proteolysis and SaAQP1o during oocyte hydration were investigated.

Materials and methods

Animals

Adult gilt-head sea bream (approximately 2 years old; 1–2 kg in body weight) were collected from South Spain and acclimated to captivity. Fish were transported to the laboratory and maintained in 12,000 l tanks under natural conditions of photoperiod and temperature, and feed three times a week with commercial pellets. Approximately three months before starting the experiments fish were fed with a mixture of pellets and squid, or with a diet manually mixed composed of 44% broodstock meal (Proaqua, Spain), 22% fresh squid, 22% fresh mussel, 11% oil mixture for broodstock feeds (Incromega), and 1.3% of vitamins (Nutreco). All procedures for the sampling of fish and sacrifice were approved by the Ethical Committee from IRTA (Spain).

Collection of ovarian follicles and induction of oocyte maturation and hydration in vitro

Groups of sexually mature males and females were separated from the broodstock stock during January (just before the natural spawning season, from February to April) and distributed into six through-flow seawater tanks (density 1.2–1.6 kg/m³; sex ratio 1:1) connected to a re-circulating water system. The temperature of the water was raised from 14°C to 18°C during 2–3 days, and photoperiod was maintained at 12 h light, 12 h dark. During the following 2 weeks, the number and timing of daily spontaneous spawnings was determined using a timetable in which the time of spawning (±30 min) was inferred from the stage of egg cleavage or embryo formation (e.g., 1 h at first cleavage, 11 h at gastrulation; 21 h primary embryo) at 18°C (Barbaro et al., 1997). The amount of eggs produced in each spawning, as well as the percentage of buoyant (viable) eggs versus sinking (non-viable) eggs, was estimated volumetrically as described (Fernández-Palacios et al., 1997). Under the thermal conditions employed, natural spawnings occurred regularly at dawn (+ 2 h) allowing to select females for sacrifice and collection of ovarian follicles at approximately 6 h before spawning, which is the time that sea bream fully-grown oocytes show the highest sensitivity to hormonal treatments by undergoing oocyte maturation in vitro (unpublished data).

At appropriate times, selected females were sacrificed and the ovary was removed and placed in Petri dishes with 75% Leovizit L-15 culture medium with 1-glutamine (Sigma) pH 7.5. Fully-grown follicle enclosed oocytes were manually isolated from ovarian pieces with watchmaker’s forceps and placed in fresh culture medium. To induce oocyte maturation and hydration in vitro, groups of follicles (n = 30–50) were incubated in culture medium with either 0.1 μg/ml of the MIS, 17α,20β-dihydroxy-4-pregnene-3-one (17,20P), or 50 IU/ml of human chorionic gonadotropin (hCG) up to 48 h at 18°C in a temperature-controlled incubator. The effect of bafilomycin A₁ (BA₁) and tetraethylammonium (TEA) on oocyte maturation and hydration was tested by adding these drugs in the medium 1 h prior to the addition of the steroid. Oocyte maturation was scored by assessing complete cytoplasmic clarification as an indicator of GVBD (Cerdà et al., 1996). In some cases, oocytes were “cleared” to confirm GVBD by treatment with 95% methanol for 1 min followed by 95% ethanol/acetic acid (3:1) (Selman et al., 2001). Changes in oocyte volume were calculated from the oocyte diameter measured with an ocular Nikon stereomicroscope to the nearest 0.01 mm until complete hydration was observed.
Water content, Na⁺ and K⁺ determination

Water content of fully-grown and mature oocytes (n = 200) was measured gravimetrically to constant weight at 60°C.

Atomic absorption spectroscopy was used to determine the concentrations of Na⁺ and K⁺ in follicle-enclosed oocytes during maturation. Groups of 15–20 follicles were placed in nitric acid-cleaned borosilicate tubes and digested in 1 mℓ HNO₃ and 0.5 mℓ H₂O₂ ("Baker Instra") for 12 h at 90°C. Samples and standards were then diluted 1:5–50 with 1% HNO₃ in deionized water and analyzed using a Unicam 939 atomic absorption spectrophotometer with Deuterium correction under standard conditions. Control (blank) tubes were treated as described above.

RT-PCR analysis of SaAQP1o gene expression

The abundance of SaAQP1o transcripts in follicle-enclosed oocytes at different stages during oocyte growth in vivo, as well as at different times during 17,20α-P- and hCG-induced oocyte maturation in vitro, was determined by reverse-transcriptase (RT)-PCR. Total RNA was extracted using the RNAeasy minikit (Qiagen), and treated with 2 U of Turbo DNase (Ambion) for 30 min at 37°C following manufacturer instructions. One microgram of digested samples was used for first strand cDNA synthesis using 0.5 μg oligo dT primer with or without 16 U of Moloney murine leukaemia virus (MMLV) RT (Roche Diagnostics). Transcripts encoding SaAQP1o were amplified from 1 μℓ cDNA using a set of oligonucleotide primers, 25 nM of dNTPs and 10 IU of Taq polymerase (Roche Diagnostics) in 50 μℓ of reaction mixture. The forward and reverse oligonucleotide primers for SaAQP1o were 5 ’-GGCGGCTCTTATCTACGATTT-3’ and 5 ’-TGAAGAGCTTTCTGCA-ACTCA-3’, respectively (Fabra et al., 2005). The PCR for ribosomal protein L6 was performed to control the variation in mRNA concentration in the RT reaction using the following forward and reverse primers: 5 ’-ATCCCCCA-AAACTCTGAAAGCA-3’ and 5 ’-AGAGCCGAGGTATCCTTTCA-3’ (Gen-Bank Accession Number DQ323508). The PCR was performed in a thermal cycler for 29 cycles, in the case of SaAQP1o, and for 24 cycles for L6, which are the cycle numbers that generated half-maximal PCR product. The PCR reaction was carried out with 1 min denaturation at 95°C, 1 min annealing at 60°C (SaAQP1o) or 62°C (L6), and 2 min extension at 72°C, with a final extension for 7 min at 72°C. The products from PCR were runned in 1.5% agarose gels and photographed.

Functional expression of SaAQP1o in Xenopus laevis oocytes

Transcription of pT-T₆-SaAQP1o constructs, and isolation, defolliculation and injection of X. laevis oocytes were done as described previously (Fabra et al., 2005). The osmotic water permeability (Pₒ) was measured from the time course of osmotic oocyte swelling in a standard assay. Oocytes were transferred from 200 mosM modified Barth’s medium (MBS) to 20 mosM MBS medium at room temperature, and the swelling of the oocytes was followed under a stereomicroscope using serial images at 2-s intervals during the first 20 s period. The Pₒ values were calculated as described (Santos et al., 2004). To examine the effect of TEA on the Pₒ, oocytes injected with 0.5 ng SaAQP1o cRNA were incubated in MBS containing different doses of the drug (0.1, 1 or 10 mM) for 15 min before the swelling assay, which was also performed in the presence of TEA.

SDS-PAGE and western blotting

Total proteins were extracted from X. laevis oocytes and sea bream ovarian follicles by homogenization in extraction buffer containing 10 mM MgCl₂, 50 mM NaCl, 20 mM Tris pH 7.6, 1 mM EDTA, and a cocktail of protease inhibitors (Mini EDTA-free; Roche) in PBS. The homogenates were centrifuged at 13,000 × g for 5 min at 4°C, and an aliquot of the clarified homogenate was kept for determination of protein concentration using the Bio-Rad Protein assay kit. The rest was mixed with 2× Laemmli sample buffer (Laemmli, 1970) and frozen at –80°C until immunoblotting.

A volume of Laemmli-mixed homogenate corresponding to 5 or 20 μg of total protein was subjected to electrophoresis on 7% or 12% SDS-PAGE. Proteins were blotted onto PVDF membranes (Bio-Rad Laboratories) in high glycin transfer buffer (190 mM glycine, 250 mM Tris, pH 8.6, 20% methanol) for SaAQP1o detection, or in 10 mM borate, 1 mM EDTA, pH 8.8 as a transfer buffer for detection of Vg-derived YPs. Membranes were blocked for 1 h at room temperature in 5% non-fat dried milk in Tris-buffered saline 0.1% Tween (TBST), and incubated overnight (1:500) with a SaAQP1o polyclonal antibody previously characterized (Fabra et al., 2005) at 4°C. For detection of sea bream Vg-derived YPs, membranes were incubated 1 h at room temperature (1:5000) with an anti-sea bream Vg antisera, which detects oocyte YPs (E. Lubzens et al., unpublished). Bound antibodies were detected with goat anti-rabbit IgG antibodies (1:8000) coupled to horseradish peroxidase using enhanced chemiluminescence (ECL detection system; Amersham). The density of the LvH band was determined using the Quantity-One software (Bio-Rad Laboratories).

Histology and immunofluorescence

For observation of morphological changes occurring during oocyte maturation and hydration, isolated ovarian follicles were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer pH 7.3 overnight. After three washes in cacodylate buffer at 4°C, 10 min each, the samples were post-fixed in 1% OsO₄ for 1 h at 4°C, washed in distilled water, and dehydrated in acetone. The samples were then embedded into Spurr resin (Spurr, 1969), and sectioned using an ultramicrotome Leica Ultracut UCT. Sections of 0.5–1 μm were stained with methylene blue and observed and photographed with a Leica DMLB light microscope. For immunofluorescence, pieces of sea bream ovary and isolated ovarian follicles, or X. laevis oocytes, were fixed in 4% paraformaldehyde in PBS, or Bouin’s without acetic acid, for 4–6 h at room temperature, and subsequently dehydrated and embedded in paraplast (Sigma). Sections of approximately 6 μm were blocked with 5% goat serum in PBST (0.1% BSA, 0.01% Tween-20 in PBS), and incubated with anti-SaAQP1o antibody (1:300–500) in PBST with 1% goat serum overnight at 4°C. In some cases, a subsequent incubation of slices with Hoechst fluorescent dye (10 μg/ml) to stain cell nucleus was carried out. After four washes, 5 min each, with PBS, the sections were incubated with FITC anti-rabbit secondary antibodies (1:300 in PBS) for 1 h, washed three times with PBS and mounted with Vectashield (Vector Labs). Using the pre-immune serum or pre-incubation of the antibody with the synthetic peptide for 1 h at 37°C previous to its application onto the sections did not reveal any staining (not shown), which demonstrated the specificity of the signals. Immunofluorescence was observed and documented with a Leica TCS-NT confocal laser-scanning microscope.

Electron and immunoelectron microscopy

Transmission electron microscopy (TEM) was carried out to examine ultrastructural alterations of yolk globules in follicle-enclosed oocytes undergoing maturation in vitro in the presence or absence of BA₁. Ovarian follicles were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, for 48–72 h at 4°C, and processed for standard electron microscopy as described (Bozzo et al., 1993). Electron micrographs were taken by using a feol EM 1010 electron microscope.

The subcellular localization of SaAQP1o in the oocyte during maturation and hydration was determined by using immunoelectron microscopy. Isolated ovarian follicles were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer, without glutaraldehyde, at 4°C overnight. After washing as described above, the samples were dehydrated and embedded into Lowiaryl K4M embedding media (Carleman et al., 1982) following the progressive lowering temperature (PLT) method by using a Leica EM AFS. Preliminary sections of 0.5 μm were carried to localize the cortical area of the oocyte, and then ultrathin sections of 70–80 nm were obtained. The sections were blocked in 0.1 M PBS pH 7.4 with 1% goat serum and 20 mM glycine for 30 min at room temperature, and subsequently incubated with the same buffer containing SaAQP1o antisera (1:10) overnight at 4°C. After four washes 5 min each, the sections were incubated with protein A-coupled to gold particles (15 nm) in 1% goat serum, 20 mM glycine PBS buffer for 1 h at room temperature. After several washes in PBS followed by distilled water, the sections were contrasted with 2% uranil acetate for 15 min and lead
citrate for 5 min. The images were documented and photographed as indicated above.

Statistical analysis

Data of P values from X. laevis oocytes expressing SaAQP1o and from in vitro incubations of sea bream ovarian follicles were statistically analyzed by the Student’s t test. Oocyte Na+ and K+ content was analyzed by one-way ANOVA. Differences were considered significant at \( P \leq 0.05 \).

Results

SaAQP1o gene expression and protein localization during oocyte growth

During the reproductive season, the gilthead sea bream presents a group-synchronous ovarian development, in which successive batches of ovarian follicles are recruited into vitellogenesis, maturation and ovulation. Thus, follicles containing oocytes at different growth stage, from previtellogenesis up to the maturation stage, can be observed in a single ovary (Figs. 1A–D). This feature allowed us to dissect ovarian follicles at different growth stage, as well as mature oocytes, and investigate the mRNA and protein expression and cellular localization of SaAQP1o in vivo.

The expression of SaAQP1o mRNA in sea bream ovarian follicles from previtellogenesis to maturation was determined by RT-PCR. The results showed the amplification of a product of 209 bp in all the stages studied, and based on the L6 mRNA internal control, the levels of SaAQP1o mRNA did not change significantly during oocyte growth and maturation (Fig. 1E). Immunoblotting analysis using the SaAQP1o antisera on total protein extracts from a mixture of ovarian follicles at different stage showed 2–3 protein bands of similar intensity with molecular masses between 27 and 29 kDa, thus corresponding to the calculated molecular mass of SaAQP1o channel (Fig. 1F, blot 1). In protein extracts from brain, in which SaAQP1o transcripts are not detected (Fabra et al., 2005), these protein bands were not observed. However, several bands with higher apparent molecular masses were detected in both brain and follicles which might suggest the cross-reactivity of the SaAQP1o antisera in Western blot with some SaAQP1o-related peptides. The immunoblotting analysis of follicle-enclosed

Fig. 1. SaAQP1o gene expression and protein localization during oocyte growth in gilthead sea bream. (A–D) Photomicrographs of follicle-enclosed oocytes at previtellogenesis (Pv) and early vitellogenesis (Ev) (A), mid-vitellogenesis (Mv, B), fully-grown (Fg, C) and maturation (M, D). (E) Representative RT-PCR for the detection of SaAQP1o mRNA during oocyte growth. The PCR for the ribosomal protein L6 was performed to control the variation in the mRNA concentration. Minus indicates the absence of RT during cDNA synthesis, and the size of transcripts (bp) is indicated on the left. (F) Western blot of protein extracts (20 μg per lane) from brain and ovarian follicles (blot 1) and from Pv + Ev, Mv, Fg and M (blot 2) using the SaAQP1o antisera. On the left, the apparent molecular masses are indicated (kDa). (G–J) Representative immunofluorescence microscopy on paraffin sections from the ovary (n = 3 females) showing follicles at Pv, Ev, Mv and Fg after reaction with the anti-SaAQP1o antisera. The sections were counterstained with Hoechst (blue color) for visualization of cell nucleus from the somatic cells associated with the oocyte. The dashed circles indicate the location of the germinal vesicle (gv). Bars, 200 μm (B–D), 100 μm (A, I and J), 50 μm (H), 20 μm (G).
oocytes from previtellogenesis up to the maturation stage indicated the presence of the SaAQP1o protein in all developmental stages, although the intensity of the SaAQP1o-reactive bands appeared to decrease as oocytes developed (Fig. 1E, blot 2).

Immunocytochemistry revealed that SaAQP1o protein was synthesized during early vitellogenesis (Figs. 1G–J). Previtellogenic ovarian follicles were devoid of SaAQP1o positive signals, while early vitellogenic follicles showed SaAQP1o immunoreaction exclusively in the oocyte cytoplasm, apparently in vesicles located at the cortical ooplasm (Figs. 1G and H). As oocytes advanced into vitellogenesis, SaAQP1o appeared to be translated towards a more peripheral area of the oocyte (Fig. 1I), and when oocytes reached the fully-grown stage, the SaAQP1o was concentrated within a thin layer just below the oocyte plasma membrane (Fig. 1J). These observations therefore indicate that SaAQP1o is synthesized de novo by the oocyte at the initiation of vitellogenesis on an already existing mRNA pool, and that during subsequent growth the protein is slowly transported towards the plasma membrane.

**Morphological changes and hydration of the oocyte during maturation in vitro**

To investigate the time-course of morphological and molecular changes of the oocyte during maturation and hydration, fully-grown follicle-enclosed oocytes were dissected from reproducing females and exposed to 0.1 μg/ml of the MIS 17,20β-P in 75% L-15 culture medium and photographed at different times. The sequential changes in the morphology of follicle-enclosed oocytes occurring during maturation were classified into four stages: fully-grown oocyte (St1), early maturing oocyte (St2), late maturing oocyte (St3), and mature oocyte (St4). Scale bar, 200 μm.

Fig. 2. Oocyte maturation and hydration in vitro in gilthead sea bream. Prematuration follicles were incubated with 0.1 μg/ml 17,20β-P in 75% L-15 culture medium and photographed at different times. The sequential changes in the morphology of follicle-enclosed oocytes occurring during maturation were classified into four stages: fully-grown oocyte (St1), early maturing oocyte (St2), late maturing oocyte (St3), and mature oocyte (St4). Scale bar, 200 μm.

The hydration occurring during oocyte maturation was quantified by determining the wet and dry weight of batches of St1 and St4 follicle-enclosed oocytes collected from different females (Table 1). When follicles matured, an 8-fold increase in their total weight was found. Although an increase of both the dry and wet weights during maturation contributed to the overall weight gain, the change in dry weight contributed less than 4% to the total weight increase and hence was trivial. The acquisition of water during maturation (from 0.05 μl in St1 to 0.51 μl in St4) was the major contributing factor to the increased weight (from 0.07 mg to 0.56 mg) of St4 follicle-enclosed oocytes. As a consequence, the relative water content of the follicles increased from approximately 63% in St1 to 92% in St4, and oocyte volume increased by approximately 350%.

**Stage-course of yolk processing during oocyte maturation**

The processes of yolk globule fusion and processing of YPs were investigated in each oocyte stage. The stage-course of fusion of yolk globules was first determined by light microscopy and TEM (Fig. 3). Prematuration oocytes at St1 showed the germinal vesicle in a central position and the yolk globules equally distributed in the cytoplasm (Fig. 3A). Yolk fusion started in St2 oocytes, coinciding with the migration of the germinal vesicle (GV) towards the animal pole of the oocyte, and by St3 most of the yolk globules appeared fused (Figs. 3B and C). As a result, a central mass of yolk is formed in St4 mature oocytes (Fig. 3D). Electron microscopy revealed the presence of numerous membrane-limited yolk globules in St1 oocytes that contained inclusions of crystalline nature (Figs. 3E and I). During St2 and St3, yolk globules fragmented and progressively fused into larger masses while their crystalline inclusions gradually disappeared (Figs. 3F, G, J and K). In mature St4 oocytes, which are translucent, a single, grown oocytes with opaque cytoplasm (Fig. 2A), stage 2 (St2) oocytes undergoing maturation and showing less than 50% of the cytoplasm with the “stippled” appearance (Fig. 2B), stage 3 (St3) maturing oocytes with more than 50% of stippled cytoplasm (Fig. 2C), and stage 4 (St4) mature, pre-ovulated oocytes showing completely translucent and hydrated appearance with a single oil droplet (Fig. 2D). The St4 was thus considered the final stage of normal maturation in vitro, prior to ovulation (Selman et al., 2001).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>St1</th>
<th>St4</th>
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<tbody>
<tr>
<td>Wet weight (mg)</td>
<td>0.07 ± 0.001</td>
<td>0.56 ± 0.01</td>
</tr>
<tr>
<td>Dry weight (mg)</td>
<td>0.03 ± 0.001</td>
<td>0.05 ± 0.002</td>
</tr>
<tr>
<td>Water weight (mg)</td>
<td>0.05 ± 0.001</td>
<td>0.51 ± 0.01</td>
</tr>
<tr>
<td>% Water</td>
<td>62.9 ± 0.9</td>
<td>91.8 ± 0.2</td>
</tr>
<tr>
<td>Volume (mm³)</td>
<td>0.16 ± 0.004</td>
<td>0.57 ± 0.008</td>
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<tr>
<td>% Volume increase</td>
<td>–</td>
<td>351 ± 5</td>
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*Values are the mean ± SEM of replicate samples (n = 200) collected from 3 to 4 females.
large membrane-bound yolk mass occupied the central ooplasm (Fig. 3H), and appeared homogeneous and less electron-dense, i.e., noncrystalline (Fig. 3L).

The ultrastructural changes of yolk globules were subsequently correlated with the MIS-activated cleavage of Vg-derived YPs (Fig. 4). The main changes of YPs typically observed by SDS-PAGE and Coomassie blue staining from St1 to St4 oocytes in gilthead sea bream is the disappearance of a YP of approximately 103 kDa, as several other smaller proteins are formed, apparently as a consequence of YP hydrolysis during oocyte maturation (Fig. 4A). Western blotting analysis using the anti-gilthead sea bream Vg antisera revealed two Vg-related YPs in St1 protein extracts of approximately 103 and 96 kDa that were named Lv heavy (LvH) and Lv light (LvL), respectively (Fig. 4A). In St4 follicles, the LvH band completely disappeared while LvL slightly decreased in size (reaching approximately 90 kDa). The Vg antisera was then employed to determine the stage-course of LvH degradation from St1 to St4 by Western blotting (Fig. 4B). The results revealed that the 103-kDa LvH band remained almost intact from St1 up to St2, in St3 its intensity became reduced, while by St4 the band was no longer detected.

Changes of Na⁺ and K⁺ during oocyte maturation

The content of inorganic ions, such as Na⁺ and K⁺, were measured during the transition from St1 to St4. Table 2 indicates that absolute amounts (nmol/individual) of K⁺ progressively increased during oocyte maturation, the highest increment being from St3 to St4. Thus, K⁺ content increased approximately 4 times during oocyte maturation. In contrast, Na⁺ content did not change from St1 to St3, and decreased by St4. Because of the pronounced hydration, however, the intracellular concentration of Na⁺ and K⁺ was reduced by 77% and 50%, respectively.

SaAQP1o trafficking during oocyte maturation

After establishing the stage-course of yolk globule fusion, proteolysis of major YPs, and K⁺ influx into the oocyte, we investigated the timing of the changes in the intracellular localization of SaAQP1o in the oocyte both by high resolution immunofluorescence and immunogold labeling (Fig. 5). In St4 oocytes, SaAQP1o fluorescence and immunogold signals were associated with the most cortical area of the oocyte (Figs. 5A, E and I), within intracellular vesicles close to the oocyte plasma membrane (Fig. 5J). When oocytes underwent maturation and advanced from St2 into St3, SaAQP1o signals showed a punctate pattern crossing the vitelline envelope or zona radiata (Figs. 5B, C, F, G, and K). Immunogold labeling on St3 oocytes revealed that this punctate pattern was due to the presence of SaAQP1o along the microvilli extending from the oocyte towards follicle cells, thus suggesting that at this stage SaAQP1o was translocated further into the oocyte plasma membrane (Fig. 5L). By St4, SaAQP1o immunostaining was
Effect of the inhibition of yolk proteolysis on oocyte hydration

Because the generation of FAAs from YPs seems to be the major contributing factor to oocyte hydration in marine teleosts (see Introduction), we therefore investigated the effect of the macrolide antibiotic BA1, a blocker of the vacuolar-type ATPase (V-ATPase) at nanomolar range, which inhibits yolk proteolysis in fish oocytes (Selman et al., 2001; Raldúa et al., 2006). Oocytes at St1 incubated with 17,20βP resumed meiosis, underwent GVBD and increased in volume by about 350% (Fig. 7A). When treated with 17,20βP in the presence of BA1, oocytes underwent GVBD but hydrated much less than those exposed to 17,20βP alone, and their cytoplasm was not homogeneously translucent (Fig. 7B). Thus, the increase in volume that normally accompanies oocyte maturation was inhibited by BA1 in a dose-dependent manner, with the I50 level being approximately 4 nM BA1 and maximum inhibition of volume increase being approximately 80% with 100 nM BA1 (Fig. 7C). Control follicles not treated with 17,20βP and those treated with BA1 alone did not undergo GVBD or change volume (data not shown).

Follicle-enclosed oocytes treated with BA1 were examined by TEM (Figs. 7D and E). Although follicles treated with 17,20βP and BA1 enlarged only minimally as they underwent maturation, some changes were observed in the structure of the yolk similar to those detected in 17,20βP-treated follicles; the crystalline inclusions disappeared and the yolk bodies fused into several masses of electron dense-material (Fig. 7E). However, these masses of yolk appeared quite different from the flocculent, electron-lucent yolk observed in St4 oocytes not treated with BA1 (compare Fig. 7E with Fig. 3L), suggesting that yolk processing was altered. Western blot analysis confirmed that yolk proteolysis was completely prevented by BA1 in a dose-dependent manner, with the I50 level being approximately 4 nM BA1 and maximum inhibition of volume increase being approximately 80% with 100 nM BA1 (Fig. 7C). Control follicles not treated with 17,20βP and those treated with BA1 alone did not undergo GVBD or change volume (data not shown).

Effect of the AQP1 inhibitor TEA on SaAQP1o water transport and oocyte hydration

The Pγ of mammalian AQP1 is inhibited by TEA, and this effect has been related to the presence of a Tyr186 residue in the

not longer detected in the microvilli, but it was still observed in vesicles at the cortical ooplasm (Figs. 5D and H).

The successive events of GV migration and breakdown, yolk processing, K+ accumulation and SaAQP1o translocation, observed in sea bream oocytes undergoing maturation and hydration in response to the MIS are summarized in Fig. 6. For this chronological representation of the data, individual measurements of oocyte volume and of the hydrolysis of LvH, expressed as changes in the density of the LvH band detected by Western blot, were included (expressed as changes in the density of the LvH band detected by Western blot of protein extracts (10 μg per lane) from ovarian follicles at St1 and St4 using an anti-sea bream Vg antiserum. The arrowheads indicate a higher and lower molecular weight bands possibly corresponding to LvH and LvL, respectively. The arrows point yolk products apparently derived from the degradation during MIS-induced oocyte maturation and hydration in vitro. A representative Western blot of protein extracts from three different follicle-enclosed oocytes at St1, St2, St3 and St4 (1 oocyte per lane) is shown. Note that the size of the LvL band progressively decreases from St1 to St4. In both A and B, molecular mass values are provided on the left in kDa.

Table 2

<table>
<thead>
<tr>
<th>Oocyte stage</th>
<th>Na+ nmol/follicle</th>
<th>mM **</th>
<th>K+ nmol/follicle</th>
<th>mM **</th>
</tr>
</thead>
<tbody>
<tr>
<td>St1</td>
<td>11.5 ± 1.0</td>
<td>90 ± 15</td>
<td>13.1 ± 0.6</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>St2</td>
<td>10.6 ± 0.6</td>
<td>71.6 ± 3.7</td>
<td>17.3 ± 1.1</td>
<td>98.7 ± 7.1</td>
</tr>
<tr>
<td>St3</td>
<td>11.6 ± 5.0</td>
<td>52.8 ± 5.4</td>
<td>22.4 ± 2.2</td>
<td>88.4 ± 9.6</td>
</tr>
<tr>
<td>St4</td>
<td>6.7 ± 1.8</td>
<td>20.2 ± 4.6</td>
<td>56.6 ± 4.9</td>
<td>55.8 ± 8.9</td>
</tr>
</tbody>
</table>

** Data are the mean ± SEM (n = 15–20) from two females. Values within the same column with different superscript are significantly different (P < 0.05).

** Assuming water volumes of 0.05, 0.13, 0.22 and 0.51 μl for St1, St2, St3 and St4 follicle-enclosed oocytes, respectively.

Fig. 4. Electrophoretic and immunological analysis of major YPs in gilthead sea bream oocytes. (A) SDS-PAGE stained with Coomassie blue and corresponding Western blot of protein extracts (10 μg per lane) from ovarian follicles at St1 and St4 using an anti-sea bream Vg antiserum. The arrowheads indicate a higher and lower molecular weight bands possibly corresponding to LvH and LvL, respectively. The arrows point yolk products apparently derived from the degradation during MIS-induced oocyte maturation and hydration in vitro. A representative Western blot of protein extracts from three different follicle-enclosed oocytes at St1, St2, St3 and St4 (1 oocyte per lane) is shown. Note that the size of the LvL band progressively decreases from St1 to St4. In both A and B, molecular mass values are provided on the left in kDa.

The successive events of GV migration and breakdown, yolk processing, K+ accumulation and SaAQP1o translocation, observed in sea bream oocytes undergoing maturation and hydration in response to the MIS are summarized in Fig. 6. For this chronological representation of the data, individual measurements of oocyte volume and of the hydrolysis of LvH, expressed as changes in the density of the LvH band detected by Western blot, were included (n = 10–15 follicles for each stage). Immediately after MIS stimulation, the GV migrated towards the animal pole of the oocyte, and starts the disassembly of yolk inclusions. Prior to GVBD, yolk globules started to fuse with one another, while subsequent hydrolysis of LvH approximately coincided with the time of GVBD, started to fuse with one another, while subsequent hydrolysis of LvH and highest K+ influx, which may create the osmotic pressure in the oocyte for SaAQP1o-mediated water transport.

Effect of the AQP1 inhibitor TEA on SaAQP1o water transport and oocyte hydration

The Pγ of mammalian AQP1 is inhibited by TEA, and this effect has been related to the presence of a Tyr186 residue in the
outer pore region in loop E of AQP1 (Brooks et al., 2000; Yool et al., 2002). Since the SaAQP1o deduced amino acid sequence also presents a Tyr201 located at the end of loop E (Fig. 8A), we tested whether TEA was able to reduce water permeability of X. laevis oocytes expressing SaAQP1o. Immunofluorescence microscopy demonstrated the translation and further translocation into the X. laevis oocyte plasma membrane of the SaAQP1o channel (Figs. 8B and C). Interestingly, two protein bands of about 28 and 29 kDa were detected by Western blot (Fig. 8D), thus resembling the situation in fully-grown sea bream oocytes (Fig. 1F, blot 2). The SaAQP1o expressed in X. laevis oocytes was able to transport water, but the Pf was reduced by about 42% by 10 mM TEA (Fig. 8E). However, a relatively low dose of TEA (0.1 mM) stimulated water transport by about 56% (Fig. 8E).

Since TEA was apparently non-toxic for sea bream oocytes, subsequent experiments were aimed to test its effect on MIS-induced oocyte hydration in vitro, following similar procedures than those previously used for BA1. Treatment of follicles with TEA did not affect oocyte maturation, but follicles matured in the presence of 1 or 10 mM TEA showed a diminished hydration when compared to those not treated with TEA (Fig. 9). In addition, unlike BA1-treated follicles,
the cytoplasm of mature oocytes treated with TEA appeared completely translucent (Figs. 9A and B). However, inhibition of oocyte hydration by TEA was lower than that observed with BA1, since 10 mM TEA only reduced oocyte swelling by approximately 20% (Fig. 9C). Interestingly, and consistent with the increased water permeability of SaAQP1o expressed in X. laevis oocytes, a weak but significant stimulation of sea bream oocyte hydration by approximately 10% was observed in response to 0.1 mM TEA (Fig. 9C).

Discussion

In several pelagophil teleosts, the extent of Lv cleavage is well correlated with the increase in FAAs and the extent of oocyte hydration during maturation, confirming the role of FAAs as important osmotic effectors for water uptake (Greeley et al., 1986; Matsubara and Sawano, 1995; Thorsen and Fyhn, 1996; Thorsen et al., 1996; Matsubara and Koya, 1997; Matsubara et al., 1999; Reith et al., 2001; Selman et al., 2001; Finn et al., 2002a,b). Accordingly, in gilthead sea bream oocytes, a pronounced proteolysis of YPs occurs during maturation, that possibly generates the increase in FAAs to drive water uptake (Carnevali et al., 1992; Ronnestad et al., 1994; Fig. 4). In barfin flounder (Verasper moseri) and haddock (Melanogrammus aeglefinus), the degradation of Vg1- and Vg2-derived Lvs during oocyte maturation is however different (Matsubara et al., 1999; Reith et al., 2001). Thus, while the heavy chain of Vg1-derived Lv is completely degraded, the heavy chain of Vg2-derived Lv undergoes a slight reduction in size, suggesting that Lv derived from Vg1 is the major source of FAAs. In barfin flounder, the heavy chains of Vg1- and Vg2-derived Lvs show molecular masses of 107 and 94 kDa, respectively (Matsubara et al., 1999), which strongly resemble the calculated molecular masses of LvH and LvL YPs in immature sea bream oocytes, of approximately 103 and 96 kDa, respectively (Fig. 4A). Although sea bream vg genes and protein products have not been fully characterized, these striking similarities suggest that the 103-kDa LvH from sea bream oocytes may correspond to the heavy chain of Vg1-derived Lv, that as in barfin flounder and haddock, becomes completely degraded during maturation. The fact that BA1 prevented the degradation of the LvH YP, and subsequent oocyte hydration (Figs. 7C and F), is in agreement with this conclusion.

The strong reduction of oocyte swelling by approximately 80% in the presence of BA1 also suggest that Lv hydrolysis has a major role during sea bream oocyte hydration as in other marine teleosts. However, although sea bream mature oocytes hydrated less in the presence of BA1, the YPs became noncrystalline and yolk globules fused with one another, thus rendering the oocyte translucent to some extent (Figs. 7B and D). These results are similar to those found in oocytes from other fish (Selman et al., 2001; Raldúa et al., 2006), suggesting that V-ATPase-mediated acidification of yolk compartments is
required for YP proteolysis during oocyte maturation. However, the oocytes of gilthead sea bream, as those from other pelagophil species, also accumulate K$^+$ ions during maturation (Table 2), thereby contributing to water uptake into the oocyte (see Introduction). In sea bream, K$^+$ influx into the oocyte began during early maturation, which is consistent with our previous hypothesis (Selman et al., 2001) that K$^+$ translocation, which is not affected by BA1, might be responsible for yolk crystal disassembly, rendering YPs available to proteases that are activated under acidic conditions (Raldúa et al., 2006).

The additional immunogold labeling and functional experiments reported here support further the role of SaAQP1o during sea bream oocyte hydration. Immunocytochemistry confirmed that SaAQP1o is synthesized at early vitellogenesis, and as oocytes develop, SaAQP1o is translocated towards the periphery of the oocyte (Figs. 1 and 5). Notably, during oocyte maturation, and preceding the periods of complete degradation of YPs and highest K$^+$ influx, SaAQP1o was translocated further into the oocyte microvilli, which was followed by the period of highest oocyte swelling (Figs. 5L and 6). The functional relevance of the changes in the subcellular localization of SaAQP1o during oocyte maturation was tested by using TEA, an inhibitor of SaAQP1o $P_f$, which is non-toxic for the oocyte unlike HgCl$_2$ (Fabra et al., 2005). Interestingly, the effect of TEA on sea bream oocyte hydration paralleled the permeability properties of SaAQP1o heterologously expressed in $X$. laevis oocytes (Figs. 8E and 9C), and therefore these observations reinforce the role of SaAQP1o mediating water uptake. The final swelling of sea bream oocytes in the presence of 10 mM TEA was reduced only to some extent, which is in agreement with the fact that TEA does not block completely AQP1 (Brooks et al., 2000) or SaAQP1o water transport (Fig. 8E). However, this observation might also indicate that water influx from blood and ovarian fluid into the oocyte can occur.
additionally by passive diffusion through the follicular (somatic cells and oocyte) membranes. Therefore, the physiological role of SaAQP1o in vivo might be the acceleration of water uptake during oocyte maturation, thus providing a highly controlled system that may be required by teleosts with group-synchronous ovaries that produce daily batches of mature (and hydrated) oocytes during the spawning season (Wallace and Selman, 1981; Kendall et al., 1984). Procedures to specifically inhibit SaAQP1o water transport, by siRNA or specific antibodies, will be needed to confirm this hypothesis.

The presence of SaAQP1 in early vitellogenic oocytes suggest that SaAQP1o synthesis may be under FSH and/or estrogen regulation, as it has been shown for several AQPs in the mammalian ovary (Richard et al., 2003; Jablonski et al., 2003). The presence of a putative estrogen binding site in the promoter region of the fugu (*Takifugu rubripes*) AQP1o ortholog, a benthophil teleost that produces hydrated eggs, supports this hypothesis (data not shown). During oocyte growth, SaAQP1o seems to be mainly regulated at the posttranslational level, since no significant variation in the amount of SaAQP1o mRNA was observed during vitellogenesis (Fig. 1E) or during hCG- or 17β-estradiol (E2) into the blood, which will induce the hepatic synthesis and release of the yolk precursors Vgs (Wallace, 1985). Circulating Vgs are incorporated by the growing oocyte, which also appears to be controlled by FSH (Tyler et al., 1991, 1997), and subsequently processed into YPs that are stored in yolk globules. As vitellogenesis begin, the SaAQP1o is synthesized and transported within vesicles towards the oocyte cortex throughout oocyte growth. When vitellogenesis resumes (Fig. 10C), oocytes are activated by the MIS synthesized by follicle cells in response to the maturation gonadotropin LH (Nagahama et al., 1995), and fusion of yolk globules and proteolytic cleavage of Vg-derived YPs is initiated. During this process, but before complete degradation of YPs occurs, SaAQP1o translocation into the oocyte plasma membrane is continued, where it mediates water transport (solid lines) driven by the increase of YP-derived FAAs and inorganic ions. As a result, a highly hydrated oocyte is produced, which will become a buoyant egg after ovulation (Fig. 10D). This model suggests that the oocyte hydration in gilthead sea bream, and possibly in other pelagophil teleosts, is

Fig. 10. Proposed model of the main physiological processes during oocyte maturation and hydration in pelagophil teleosts. (A) Follicle cells associated to previtellogenic oocytes are stimulated by FSH to produce E2, which will induce the hepatic synthesis and release of Vg into the blood. (B) During oocyte growth (vitellogenesis), Vg is incorporated by the oocyte by a receptor-mediated endocytosis, a process that also appears regulated by FSH, and subsequently processed into YPs and stored into yolk inclusions. At the initiation of vitellogenesis, the AQP1o is synthesized and transported within vesicles towards the oocyte plasma membrane. (C) At maturation stage, the granulosa cells are stimulated by LH to produce the MIS, which will induce meiosis resumption of the oocyte. During this process, the GV migrates towards the animal pole and eventually breaks down, yolk globules fuse with one another and YP hydrolysis is activated, while inorganic ions, such as K+ and others (e.g., Cl− (Finn et al., 2002a)), are accumulated in the oocyte. At this time, AQP1o is further translocated into the oocyte plasma membrane, where it can mediate water transport (solid lines) driven by the increase of YP-derived FAAs and of other small-molecular-weight osmotic effectors. Additionally, water influx into the oocyte can occur by simple diffusion through the follicular membranes (dotted lines). (D) When yolk proteolysis is completed, AQP1o-containing vesicles are retrieved from the oocyte plasma membrane. As a result, a highly hydrated oocyte is produced.
Acknowledgments

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References


